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TITLE: PRODUCTION OF CYTOKINE-SPECIFIC MONOCLONAL ANTIBODIES THAT MODULATE IMMUNE AND INFLAMMATORY PROCESSES

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13. ABSTRACT *(Maximum 200 words)*

The goal of this cooperative agreement is to generate, characterize and produce substantial quantities of monoclonal antibodies (mAb) that enhance protective actions of murine cytokines. During the past funding period, we have used roller bottle and bioreactor tissue culture techniques and large scale HPLC Protein A affinity chromatography techniques to establish effective scale-up procedures that have resulted in the purification to homogeneity of 200+ mg quantities of mAb in aggregate and endotoxin free form. Four hundred mg of assorted purified mAb have already been provided to the Army for research purposes. This accomplishment thereby completes the first specific aim of the project. In addition, a new family of hamster mAbs have been generated that are specific for either the p55 or p75 murine TNF receptors. Within this family of antibodies are mAb the either inhibit or mimic TNF action i.e. which function either as TNF antagonists or agonists. These antibodies have been partially characterized using standard laboratory protocols and currently are being tested for beneficial protective effects in murine model systems. This result thus places us well along in completing the second specific aim thereby putting us right on schedule for the overall project.

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Robert D. Schmidt 4/13/94

PI - Signature Date

TABLE OF CONTENTS

I. INTRODUCTION

The basic hypothesis that underlies this cooperative agreement is that monoclonal antibodies can be generated that enhance the protective actions of a particular cytokine and thereby confer upon the host an increased nonspecific resistance to a variety of disease causing infectious agents such as viruses, bacteria, and parasites. To test this hypothesis we put forth three specific aims in the original proposal. First, we planned to develop the capability of producing substantial amounts of several of our existing hamster anti-murine cytokine monoclonal antibodies under tightly controlled, tissue culture conditions and then purify them to homogeneity in aggregate-free and endotoxin-free form. Moreover, we agreed to provide 400 mg of purified antibody each year to Army scientists for research purposes. *This first specific aim has been achieved during the first year funding period.* The second specific aim was to generate cytokine specific monoclonal antibody reagents that enhance specific cytokine activities in an attempt to augment the activity of endogenously produced cytokines elaborated during immune or inflammatory responses and thereby promote host defense processes. *We have made good progress on this specific aim and have generated a novel family of monoclonal antibodies specific for the two murine TNF receptors which have either agonist or antagonist activities.* Finally the third specific aim was to produce monoclonal antibody reagents that selectively inhibit only certain biologic activities of a cytokine but not other activities in order to selectively enhance the ability of a cytokine to promote host defense reactions while at the same time ablating its toxic or immunopathologic effects. *This aim will be addressed in year three of the project. However, we believe that the anti-TNF receptor monoclonal antibodies we have generated may provide us with a mechanism to separate positive versus negative effects of certain cytokines.*

II. NARRATIVE OF PROGRESS

A. Specific Aim 1

We have made excellent progress on the first specific aim and now consider that our first objective has been achieved. The target goal for the first year of this award was to develop the capability within the lab to generate substantial quantities of highly purified, contaminant free monoclonal antibodies. We had previously produced a family of hamster hybridomas that produced neutralizing monoclonal antibodies against murine IFNγ, TNF and IL-1 (1-6) and therefore we decided to use these cell lines for developing a large scale production protocol.

Two procedures were used to generate large quantities of antibody in crude form that were suitable for subsequent purification. The first was based on the conventional protocol of generating antibody containing culture supernatants in roller bottles. We adapted our hybridoma cell lines to grow in roller bottles

and then recloned them to isolate high antibody producers. Then each cell line was expanded into 10-20 liters of culture supernatant and grown to stationery phase. Depending on the particular cell line, the spent culture supernatants were found to contain between 10-25 mg mAb/liter thus permitting us the capacity to generate between 100-500 mg of each antibody in crude form in approximately 4 weeks. The second protocol was based on the use of Amicon Bioreactors. To date three separate bioreactors have been tested and the protocols for their set up and maintenance established. Two bioreactors have been extremely successful and have yielded 600 and 700 mg of crude antibody, respectively, The third developed a leak early in the run and therefore only 150 mg of antibody was obtained. Bioreactors have significant advantages over the roller bottles since (1) antibody concentrations are 30-100 times higher in bioreactor fluids versus roller bottle supernatants (antibody concentrations in the bioreactor taps (10 ml/day) are between 0.3-1.0 mg/ml whereas they are 10-25 μg/ml in 1 liter roller bottle derived culture supernatants), (2) it is easier to store the smaller volumes generated in the bioreactor before purification, and (3) the small volume of bioreactor supernatants reduces the potential for subsequent endotoxin contamination of the protein A column used for purification.

Large scale antibody purification has been achieved using a 5.0 x 12.0 cm column packed with Protein A Affiprep (Bio-Rad Laboratories, Richmond, CA) under endotoxin free conditions. If necessary, antibody containing culture supernatants are concentrated in an Amicon spiral concentrator, cleared by centrifugation and then pumped onto the Protein A Affiprep column equilibrated in 0.05M borate buffer/0.15M NaCl using a Waters 650 HPLC system adapted with a peristaltic pump for sample application. The column is washed with 10 volumes borate buffer and then antibody is eluted using 0.05M glycine-HCl/0.15M NaCl pH 2.0. The fractions are collected into plastic tubes containing 1.0M Tris buffer pH 8.5 and analyzed for protein by monitoring absorbence at A280 nm. Antibody-containing fractions are pooled, dialyzed, sterile filtered and frozen. We now routinely use this protocol to produce 200-400 mg quantities of purified antibody per run.

The development of this antibody production protocol allowed us to meet our commitment of supplying the Army with 400 mg of highly purified cytokine specific monoclonal antibodies during the first year of the award. The specific antibodies delivered are as follows:

Antibody	Specificity	Quantity	Date Shipped
H22	Murine IFNγ	100 mg	9/16/92
TN3-19.12	Murine TNFα	100 mg	9/16/92
H22	Murine IFNγ	100 mg	7/19/93
TN3-19.12	Murine TNFα	100 mg	7/19/93
Total Antibody		**400 mg**	

B. Specific Aim 2

Progress has also been made on exploring whether we can produce a monoclonal antibody that can enhance the actions of a cytokine. The initial plan was to focus on the antibody we already had that appeared to enhance the action of IFNγ *in vitro* (1) and we indeed still plan to proceed with that objective during the coming second half of the funding period. However, we also wondered whether we could generate a monoclonal antibody specific for a cytokine receptor that had agonist activity and could thereby mimic a specific cytokine action. We chose to explore this possibility during the first half of the funding period and we now report that we have indeed generated both agonist and antagonist monoclonal antibodies specific for each of the two murine TNF receptors (Sheehan, K. C. F., Pinckard, J. K., Arthur, C., and Schreiber, R.D. Manuscript in preparation). These are extremely unique antibodies since at the present time they represent the only reagents in existence that can detect the murine receptor proteins. We anticipate characterizing these antibodies more fully during the coming year. A summary of the antibodies is enumerated below.

Two panels of monoclonal antibodies (mAb) specific for either the 55- or 75-kDa murine TNF receptor were generated by immunizing Armenian hamsters with purified extracellular domains of either protein (7,8). Antibody producing cultures were identified by immunoprecipitation assays that employed radiolabeled extracellular receptor domains and were then used to immunoprecipitate the appropriate receptor from cell surfaces (Fig. 1). All mAb reacted only with the specific form of the receptor used for immunization. *In vitro* analyses demonstrated that several mAb specific for the 55 kDa TNF receptor, (55R-170.1,-176.11 and -329.1) blocked the ability of either murine or human TNF to effect killing of L929 cells (2), while another, 55R-286, had no effect (Fig. 2). However, when subsequently crosslinked with anti-hamster Ig, the antagonistic mAb displayed agonist activity in the absence of exogenous TNF (Fig. 3). In addition, a fourth anti-p55 mAb (55R-593.4) induced cytocidal activity in the absence of crosslinking. In contrast, none of the mAb specific for the 75 kDa receptor (TR75-4.6, -32.4, -45.30, -54.7, -89) displayed agonist activity for cell killing either alone or following crosslinking. Although three of the anti -p75 mAb (TR75, -32.4, 45.30, and 54.7) inhibited the ability of murine TNF to kill L929 cells, they had no effect on killing induced by human TNF (Fig. 2). These results are consistent with the concept that p75 serves to focus murine TNF at the target cell surface and then "passes" the focused ligand to p55 which is responsible for initiating signal transduction events (9,10).

The *in vivo* effects of these mAb were monitored using a model of endotoxin shock (11). C57BL/6 mice were pretreated with mAb or saline and then challenged 18 hr later with LPS (600 ng) and galactosamine (8 mg). The survival of the TR75-treated mice was indistinguishable from the saline controls (‹20%) (Fig. 4). In contrast, all of the 55R-treated animals survived. This data

thus supports the concept that the two TNF receptors induce unique arrays of biologic responses and confirms that this functional division occurs *in vivo* as well as *in vitro*.

III. CONCLUSIONS AND PLANS FOR THE COMING YEAR

The project is precisely on schedule and we have now developed the ability to produce substantial amounts of antibody is highly purified form. In addition, we now have two candidate antibodies (one specific for IFNγ and one for the type I TNF receptor) that should allow us to examine whether they can indeed enhance the actions of a particular cytokine *in vivo*. Moreover, with the array of anti-TNF receptor monoclonal antibodies that we have now developed we should be in a good position to begin the work on the third specific aim of this cooperative agreement i.e. to determine whether we can produce a therapeutic modality that will permit the desirable actions of a cytokine while at the same time inhibit its pathobiologic actions. TNF will be the obvious target of this investigation.

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V. APPENDIX

Figure Legends

Figure 1: *Anti-p55 and Anti-p75 Immunoprecipitation of TNF receptors from L929 cells.* Murine L929 lysates were precipitated using 10 μg of p55 or p75 specific mAb. Material representing 1 x 10^7 cells were run on 12% SDS-PAGE under non-reducing conditions, transferred to nitrocellulose, blocked and stained with 2 μg/ml either biotin-conjugated 55R-593 (top) or TR75-54 (bottom). Bands were detected using Streptavidin-HRP (1:4000) and ECL autoradiography. 55R-286, a non-blocking p55 specific mAb similarly immunoprecipitated a specific band of 55 kDa (data not shown). TR75-4 and TR75-89, both reactive with p75 were also capable of precipitating doublets of 70 and 60 kDa (data not shown).

Figure 2. *Inhibition of TNF Cytolytic Activity by p55 and p75 TNF Receptor Specific mAb.* L929 cells are incubated with various concentrations of control Ig, anti-p55 (A and B) or anti-p75 (C and D) for 2 hours at 37°C prior to addition of 10 units/well of either MuTNFα (A and C) or HuTNFα (B and D). The % monolayer viability is expressed as described above. Untreated control monolayers displayed an absorbence of 1.52. Incubation of monolayers with 10 units murine or human TNF produced optical densities of 0.316 and 0.218, respectively.

Figure 3: *Agonistic Activity of Anti-p55 mAb.* Various dilutions of anti-p55 (A and B), anti-p75 (C and D) or control Ig 6C8 were incubated overnight with actinomycin D treated L929 in the absence (A and C) or presence (B and D) of goat anti-hamster Ig (20 μg). Monolayer viability was assessed by vital dye uptake and expressed absorbence at 540 nm. Control monolays produced an optical density of 1.12, while those containing only hamster Ig produced readings of 1.15.

Figure 4. *Protection from LPS-Galactosamine Mediated Lethal Shock by p55-Specific mAb.* Groups of 10 C57B1/6 mice were treated with saline, TNF or mAb 18 hours prior to challenge with LPS (600 ng) combined with D-galactosamine (10 mg). Survival was monitored over a 48 hour period.

IMMUNOPRECIPITATION OF MURINE TNF RECEPTORS FROM L929 CELLS

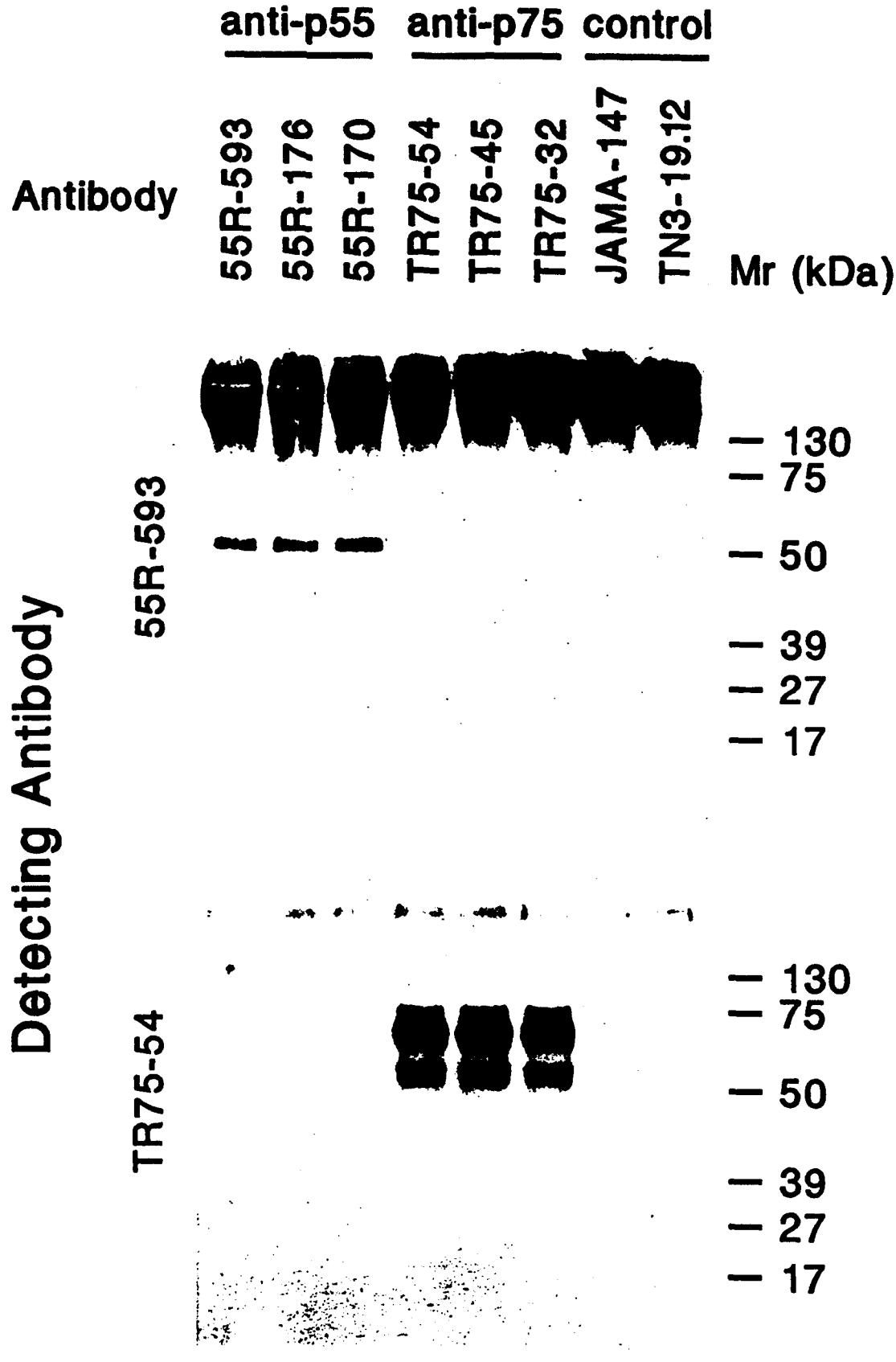

FIGURE 1

INHIBITION OF TNF CYTOLYTIC ACTIVITY BY TNF RECEPTOR SPECIFIC MONOCLONAL ANTIBODIES

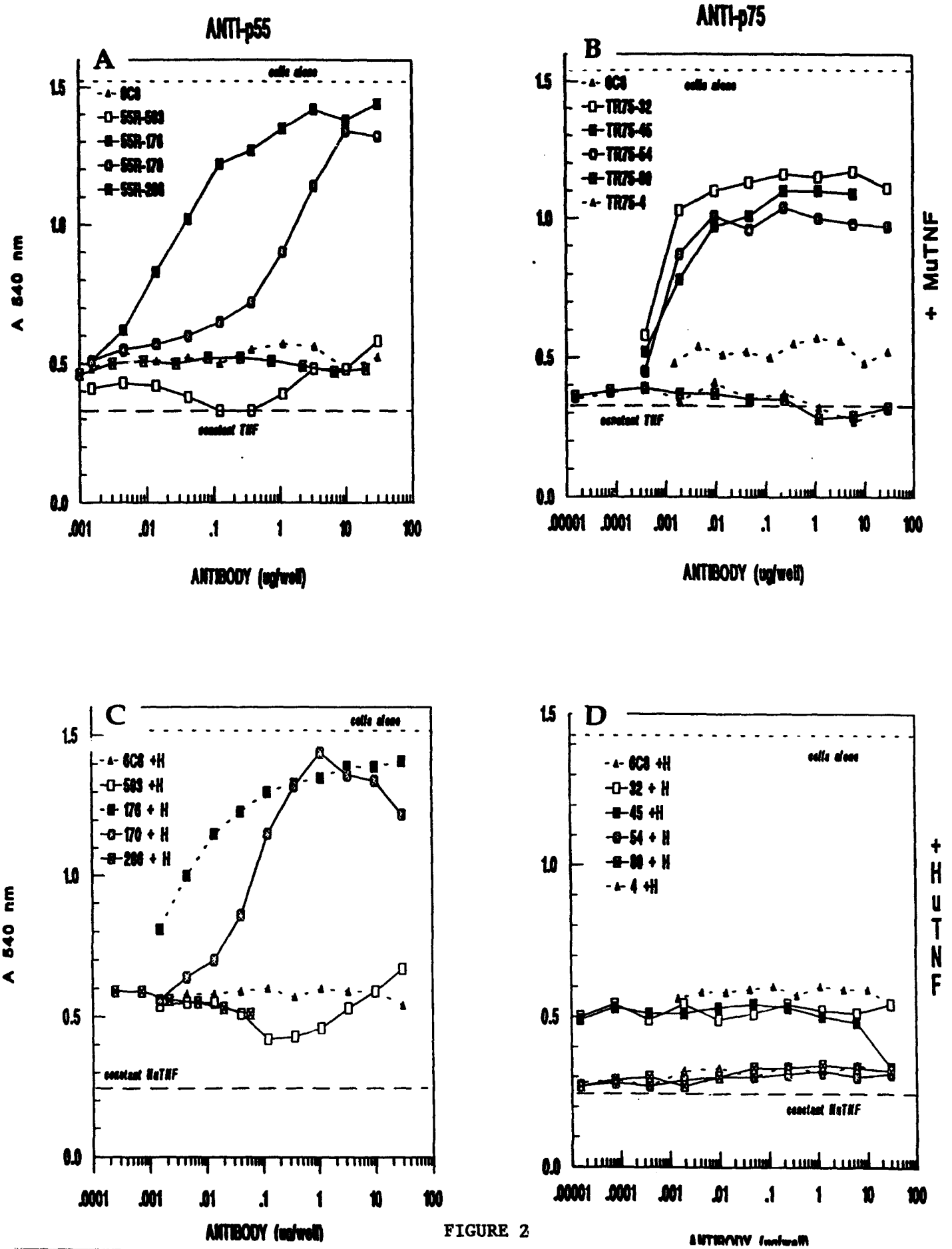

FIGURE 2

AGONIST ACTIVITY OF p55-SPECIFIC TNF RECEPTOR MONOCLONAL ANTIBODIES

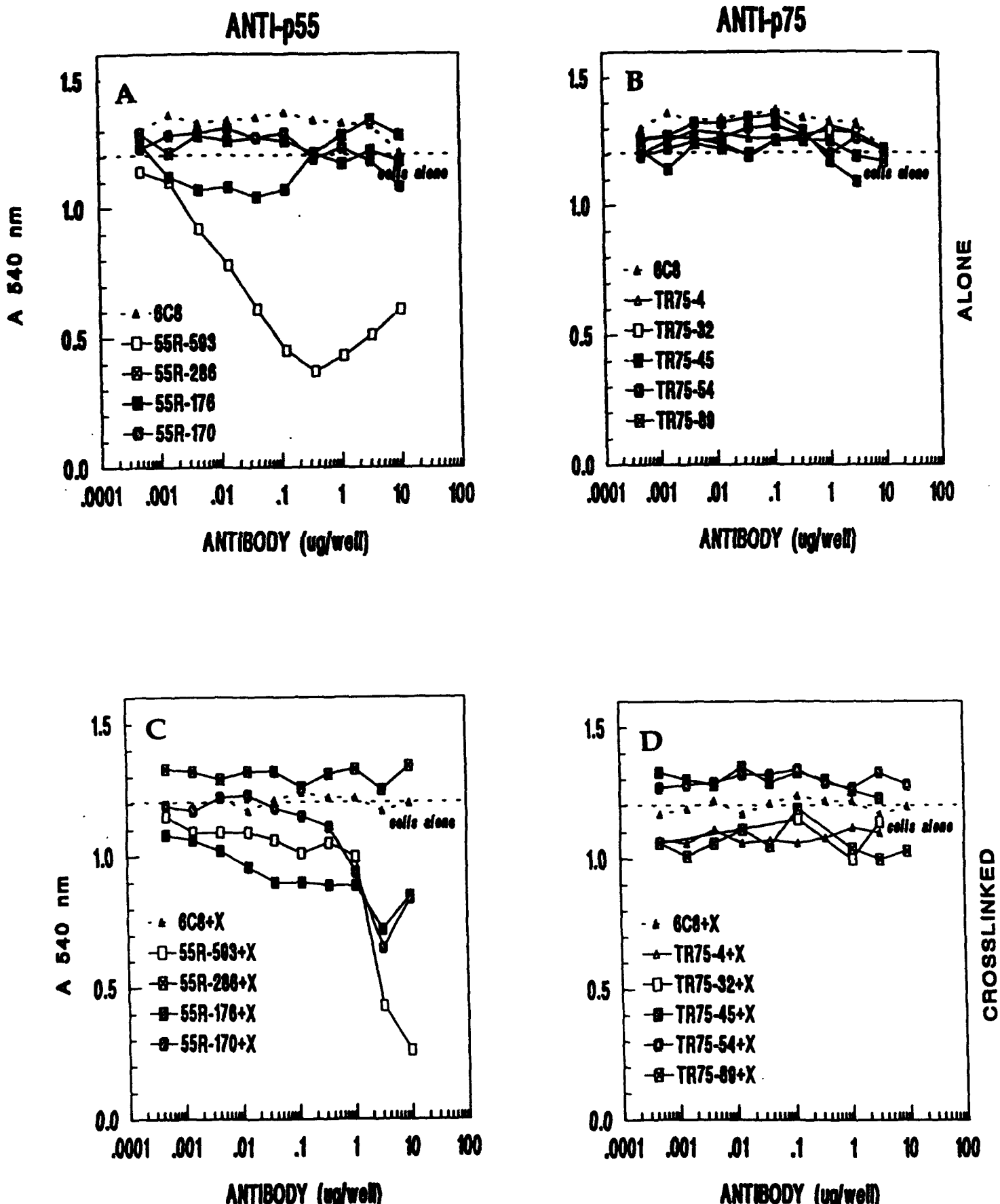

FIGURE 3

PROTECTION FROM LPS-GALACTOSAMINE INDUCED LETHAL SHOCK IS MEDIATED BY p55-SPECIFIC TNF RECEPTOR mAb

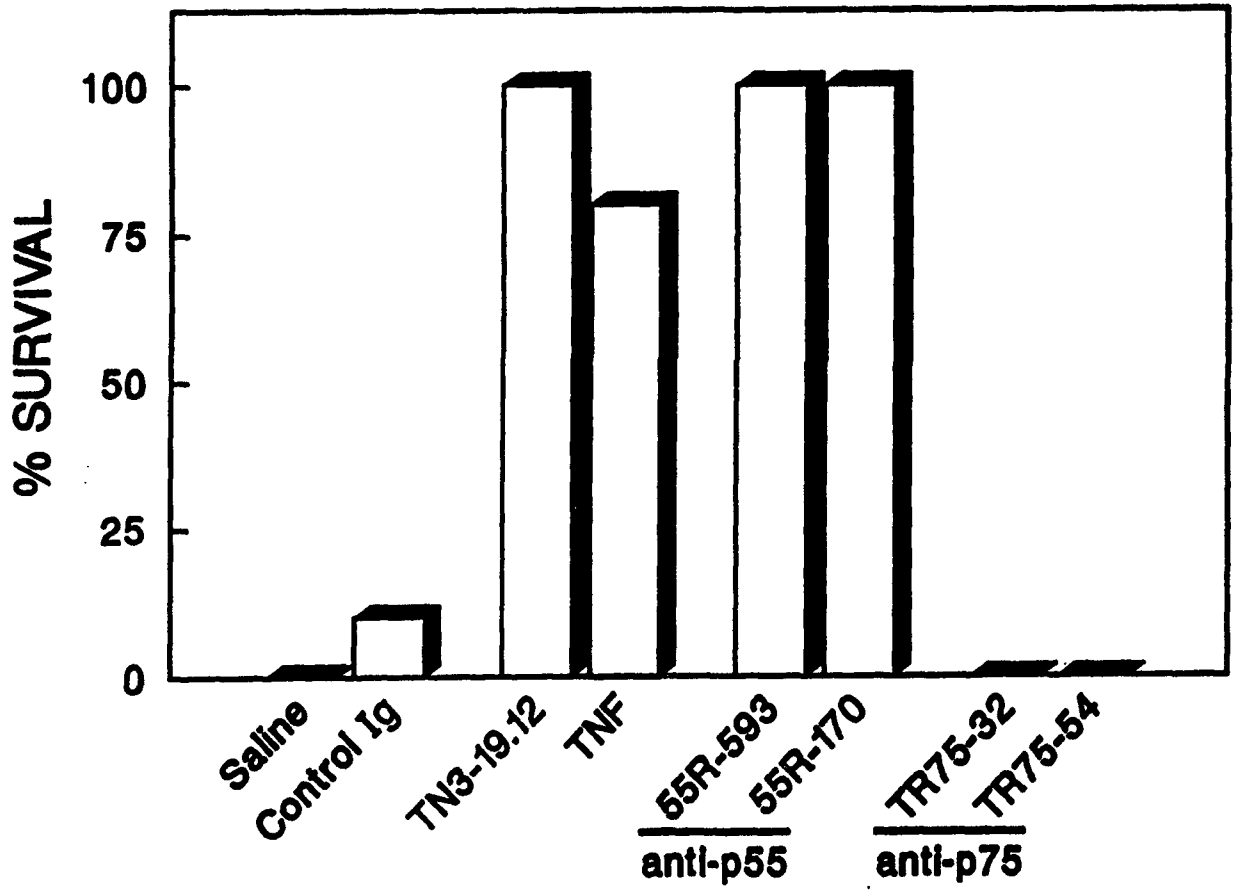

FIGURE 4